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Sperm sample collection and sRNA sequencing

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Sperm microRNAs confer depression susceptibility to offspring

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Detailed protocol

Sperm sample collection and sRNA sequencing

Abstract

Sperm RNA is increasingly recognized as an additional source of paternal hereditary information beyond DNA (*1-3*). Sperm carry a population of sRNAs, including miRNAs, PIWI-interacting RNAs (piRNAs) and tsRNAs (*4, 5*). To identify which specific subtypes of sperm sRNAs cause abnormalities in offspring, we examined the sRNA profiles of sperm derived from mice by RNA deep sequencing. The following protocol describes approaches for collecting mature sperm from the cauda epididymis of male mice of the C57BL/6J background, sRNA library construction and sequencing, and data processing and analysis.

Keywords: sperm collection; sRNA; sequencing; data processing and analysis

Background

In our previous work, we show that F1 offspring born to F0 males of depression-like model are susceptible to depression-like symptoms at the molecular, neuronal and behavioral levels. Sperm small RNAs recapitulate paternal depressive-like phenotypes in F1 offspring (*6*). To identify which specific subtypes of sperm sRNAs cause abnormalities in offspring, we examined the sRNA profiles of sperm derived from F0-Dep and F0-Ctl by RNA deep sequencing (*6*).

Materials and Reagents

PBS buffer (130 mM NaCl, 10 mM Na_2PO_4, 1.7 mM KH_2PO_4, and 2 mM KCl, pH 7.4)
Somatic cell lysis buffer (0.1% SDS, 0.5% Triton X in DEPC H_2O)
TRIzol Reagent (Invitrogen)
DEPC H_2O (Invitrogen)
10 cm dish (Corning)
DNase/RNase-free Tubes (1.5 mL; Axygen)
Sterile Conical tubes (15 mL; Corning)
DNase/RNase-free plastic tips (10 μL, 200 μL and 1,000 μL; Axygen)
Chloroform (HUSHI, Shanghai, China)
Ethanol, 75% (HUSHI, Shanghai, China)
Isopropanol (HUSHI, Shanghai, China)
40 μm cell strainer (Biofil, GuangZhou, China)
NEB Small RNA Sample Pre Kit (NEB)
14-30 ssRNA Ladder Marker (TAKARA)

Equipment

Microscopy (Olympus)
Refrigerated centrifuge (Thermo Fisher Scientific)
Micropipettes (Eppendorf)
CO_2 gas incubator (Thermo Fisher Scientific)
Vortex-Genie 2 (Scientific Industries)
Magnetic bead homogenizer (Shanghai Jingxin, Shanghai, China)
Nano Drop (Thermo Fisher Scientific)
Agilent 2100 bioanalyzer (Thermo Fisher Scientific)

Procedure

A. Sperm isolation

Mature sperm were extracted from the cauda epididymis of male mice of the C57BL/6J background and processed for RNA extraction as previously

described (*5, 7*).

1. Euthanize the 8-10 week male C57BL/6J mice and use fine scissors and forceps to dissect the cauda epididymis, removing it from the adipose tissue.
2. Place the epididymis in 5 ml preheating PBS buffer in 10 cm dish, use the syringe to puncture the epididymis.
3. Incubate the punctured epididymis for 15 min in a CO_2 incubator at 37 °C.
4. Remove the epididymis, then filter the buffer with a 40 μm cell strainer to get rid of the tissue debris.
5. Centrifuge the buffer at 600 g for 5 min, and remove the supernatant by micropipetting.
6. Treat the centrifugated deposit with somatic cell lysis buffer (0.1% SDS, 0.5% Triton X in DEPC H_2O) for 40 min on ice to eliminate somatic cell contaminants.
7. Centrifuge the buffer for 5 min at 600 g at 4°C, and remove the supernatant by micropipetting.
8. Add 10 ml of PBS and then centrifuge the buffer for 5 min at 600 g at 4°C, remove the supernatant and get centrifugated deposit.
9. Repeat step 8, and get the sperm pellet.
10. The purity of sperm was evaluated by examining morphology under light microscopy or confirmed by RT-PCR analyses of different biomarkers as previously described (*5*).

B. Total RNA extraction

Total RNA of sperm was isolated using the TRIzol Reagent according to the manufacturer's instructions.

1. Add 1 mL of TRIzol Reagent into the sperm pellet, and samples were homogenated by Magnetic bead homogenizer for 60s in 60Hz.
2. Incubate for 5 minutes to permit complete dissociation of the nucleoproteins complex.
3. Add 0.2 mL of chloroform, then securely cap the tube.
4. Incubate for 3-5 minutes.
5. Centrifuge the sample for 20 minutes at 16,000 at 4°C.
6. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and petting the solution out.
7. Add 0.8 ml of isopropanol to the aqueous phase.
8. Incubate overnight at 4°C.
9. Centrifuge for 10 minutes at 16,000 at 4°C.
10. Discard the supernatant by micropipetting.
11. Resuspend the pellet in 1 mL of 75% ethanol.
12. Vortex the sample briefly, the centrifuge for 20 minutes at 16,000 at 4°C.
13. Discard the supernatant by micropipetting.
14. Vacuum or air dry the RNA pellet for 5-10 minutes.
15. Resuspend the pellet in 30 μl of RNase-free water.

C. sRNA library construction and sequencing

The prepared RNA for Mature sperm were shipped to the Beijing Genomics Institute (BGI), Shenzhen, China in dry ice for small RNA library construction and Solexa high-throughput sequencing followed their standard protocols.

1. The Method of Sample Detecting

Use Agilent 2100 Bioanalyzer to test sample integrity and concentration, and NanoDrop to Inorganic ions or polycarbonate contamination. This step aimed to provide a reference for library construction and later analysis.

2. Library Construction

According to the NEB Small RNA Sample Pre Kit (NEB), we prepare the libraries follow as:

a. Filter Small RNA: Use the 200ng-1ug of RNA sample, then separate RNA segment of different size by PAGE gel, select 18-30nt (14-30 ssRNA Ladde Marker, TAKARA) stripe and recycle;

b. Adaptor ligation: Prepare connection 3'adaptor system (NEB Small RNA Sample Pre Kit); (Reaction condition: 70°C for 2min; 25°C for 1h); Secondly adc RT-Primer, (Reaction condition:75°C for 5min; 37°C for 15min; 15°C for 25min); Thirdly add 5'adaptor mix system (Reaction condition: 70ºC for 2min; 25ºC for 1h);

c. RT –PCR: Prepare First Strand Master Mix and Super Script II (Invitrogen) reverse transcription (Reaction condition: 70°C for 2min; 50ºC for 1h); Severa rounds of PCR amplification with PCR Primer Cocktail and PCR Master Mix were performed to enrich the cDNA fragments (Reaction condition: 94ºC fo 30s; 11-13 cycles of (94ºC for 15s, 62ºC for 30s, 70°C for 15s); 70ºC for 5min; 4ºC hold);

d. Purify PCR products: Then the PCR products were purified with PAGE gel. Dissolve the recycled products in EB solution.

3. Validation of the Library

The final library was quantitated in two ways: Determine the average molecule length using the Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 Reagents), and quantify the library by real-time quantitative PCR (QPCR) (EvaGreen).

4. Sequencing Libraries

The qualified libraries were amplified on cBot to generate the cluster on the flowcell. And the amplified flowcell was sequenced single end on the Illumina System, read length 50 was the most frequently used sequencing strategy.

After library quality validation, raw data for each sRNA library were generated on the Illumina HiSeq 4000 platform. Sequence reads that fit any of the following standard quality control criteria parameters were removed: *i*) reads with N (more than 4 bases whose quality score is lower than 10 or more than 6 bases whose quality score is lower than 13); *ii*) reads with 5' primer contaminants or without a 3' primer; *iii*) reads without the insert tag; *iv*) reads with ploy A; and *v*) reads shorter than 18 nt. The clean reads were obtained after data filtration.

D. Data processing and analysis

1. Precursor and mature miRNA sequences, tRNA sequences, piRNA sequences and rRNA sequences were obtained from miRBase v21, GtRNAdb

1. Precursor and mature miRNA sequences, tRNA sequences piRNA sequences and rRNA sequences were obtained from miRBase v21, GtRNAdb piRNA bank and NCBI respectively.
2. Bowtie (version 1.2.2) was used to align clean reads to these reference sequences for annotation.
3. To annotate miRNA, only candidates with 1 mismatch and no more than 2 shifts were counted as miRNA matches.
4. To annotate piRNA, only candidates with 1 mismatch were counted as piRNA matches.
5. To annotate tsRNA and rsRNA, SPORTS 1.1 (*8*) based on bowtie was used for tsRNA and rsRNA annotation.
6. For normalization, the total sequencing frequency of each type of sRNA in each sample was normalized to 1,000,000. Differential analysis was performed using Student's t test.
7. Significance was set at uncorrected $P < 0.05$ for broad pattern identification. A fold-change threshold was set at > 2. The average expression level threshold was set at > 500.

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